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EXPERIMENTAL ARTICLES

Microbiological Analysis of Cryopegs from the Varandei Peninsula, Barents Sea

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Abstract—The paper deals with the microbiological characterization of water-saturated horizons in permafrost soils (cryopegs) found on the Varandei Peninsula (Barents Sea coast), 4–20 m deep. The total quantity of bacteria in the water of cryopegs was 3.5×10^8 cells/ml. The population of cultivated aerobic heterotrophic bacteria was $3–4 \times 10^7$ cells/ml and the number of anaerobic heterotrophic bacteria varied from 10^2 to 10^5 cells/ml depending on cultivation temperature and salinity. Sulfate-reducing bacteria and methanogenic archaea were found as hundreds and tens of cells per ml of water, respectively. A pure culture of a sulfate-reducing strain B15 was isolated from borehole 21 and characterized. Phylogenetic analysis has shown that the new bacterium is a member of the genus *Desulfovibrio* with *Desulfovibrio mexicanus* as its closest relative (96.5% similarity). However, the significant phenotypic differences suggest that strain B15 is a new species of sulfate-reducing bacteria.

Key words: cryopegs, permafrost, psychrophilic bacteria, sulfate-reducing bacteria, *Desulfovibrio* sp., *Psychrobacter* sp., *Pseudomonas* sp.

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Cryopegs are non-frozen soils and lenses of free water which occur within permafrost and are characterized by constant subzero temperatures. These formations are widespread in the Arctic. The origin of cryopegs is associated with frost penetration into deposits of marine genesis of different ages; their salinity is determined primarily by temperature. The involvement of surface fresh water in the formation of some cryopegs also has a significant effect on their mineralization. Our previous microbiological studies of the lenses of highly mineralized (170 g/l) cryopegs in the region of Lake Yakutskoye of the Kolymskaya lowland have shown that these ecosystems are inhabited by a psychrophilic halotolerant microbial community [1]. New taxa of psychrophilic and psychrotolerant bacteria have been isolated from these samples and characterized [2, 3]. Study of the physiological and biochemical properties of the isolates has shown that the bacteria not only survive under negative temperatures and high salinity of the environment, but also remain metabolically active [4, 5]. The number of sulfate-reducing bacteria in these cryopegs was as high as 10^6 cells per ml of water; however, we failed to obtain pure cultures of these prokaryotes.

The goal of the present work was to determine the quantity of bacteria of different physiological groups and to search for new sulfate-reducing bacteria in the samples of water-saturated horizons of the Varandei Peninsula.

MATERIALS AND METHODS

The region and object of study. The research was carried out on the Varandei Peninsula (Barents Sea coast) in August 2004. Figure 1 presents the layout of the boreholes used for sampling for the microbiological study.

Permafrost in the area of research was of three types: an upper layer of newly formed (i.e. frozen in the last five–six thousand years) permafrost soil, 4–5 m thick (Fig. 1, *1*); a layer of cooled water-saturated saline deposits (Fig. 1, *2*); and relic permafrost of the Pleistocene age (Fig. 1, *3*). The latter may also contain cryopeg lenses. The temperature of the upper frozen horizon in summer does not exceed –2°C within the coastal

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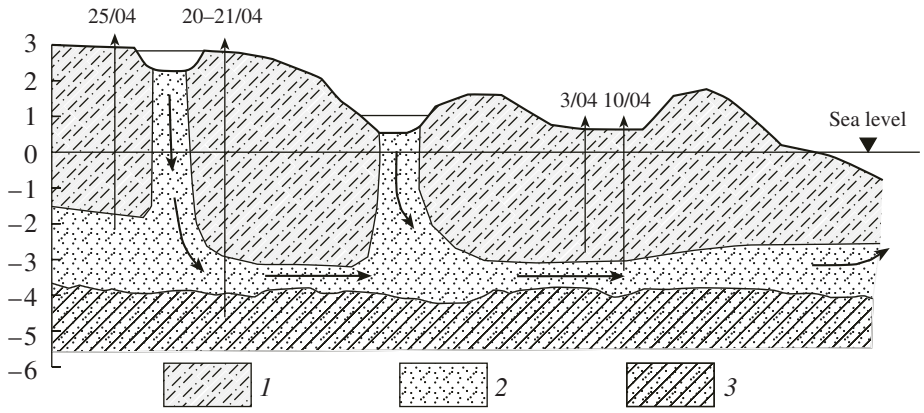

Fig. 1. The layout of the boreholes in the research area: *1*, sands of coastal–marine genesis; *2*, alternation of sands and clay sands; *3*, clay sands of marine origin.

bank and –3°C on the marine terrace. The area on the whole is characterized by average annual temperature of soils from –2 to –5°C.

The upper permafrost horizon was exposed by two boreholes within the bank (3 and 10) and two boreholes (21 and 25) on the marine terrace. The section is represented by sands of the coastal–marine genesis with silty deposit lenses. All deposits were saline and contained methane (0.2–12 ml/kg in sands and up to 22 ml/kg in silty deposits). Therefore, the conditions of their formation were probably anaerobic.

The interpermafrost horizon of pressure subsurface waters was exposed by all boreholes at the depth of 4–6 m on the bank and 4–8 m on the marine terrace. Total mineralization was 6 to 18 g/l. Such low water salinity was apparently due to desalination of the horizon as a result of the infiltration of atmospheric precipitation and surface waters via sublake taliks. The waters contain mainly sodium chloride, and only one borehole was shown to contain sodium sulfate (Table 1). The waters had neutral pH values (6.8–7.8) and low content of O_2 (0.3–0.7 mg/l).

Sampling. The methods of sterile sampling upon core boring of frozen soils by an UKB-12/25 rig have been thoroughly elaborated [6, 7]. The medium for enrichment cultures was inoculated with cryopeg waters immediately after sampling. Selected samples were stored frozen prior to analysis.

Medium composition and methods of enumeration of bacteria. The quantity of microorganisms was determined by total counts on erythrosine-stained filters. Aerobic heterotrophic bacteria were enumerated on 5/5 agar medium containing the following: aminopeptide, 60 ml; tryptone, 5 g; yeast extract, 1 g; soy extract, 30 ml; agar, 20 g; distilled water, 1 l. The quantity was determined by counting the colonies grown by day 20 and 40 (–2°C); by day 14 and 21 (5°C); by day 5 and 10 (20°C); and by day 3 and 6 of incubation (37°C).

The quantity of viable anaerobic bacteria of different physiological groups was determined by the most probable number (MPN) method. Incubation temperatures were 5 and 20°C. Bacterial numbers were determined after 14 and 21 days (incubation at 20°C) and after 45 and 60 days (at 5°C). The quantity of anaerobic heterotrophic bacteria with fermentative metabolism was determined on the medium containing (g/l): KH_2PO_4, 0.7; NaCl, 1.0; NH_4Cl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; Na ascorbate, 1.0; glucose, 2.0; peptone, 2.0. Growth was detected by increase in OD at 600 nm.

Sulfate-reducing bacteria were enumerated on the medium containing the following (g/l): KH_2PO_4, 0.33; KCl, 0.33; NaCl, 2.0; NH_4Cl, 0.33; $MgCl_2$, 0.33; $CaCl_2$, 0.33; Na_2SO_4, 4.0; solution of trace elements SL-10 [8], 10 ml; vitamin solution [9], 5 ml; lactate, 2.0. Growth was detected by hydrogen sulfide production.

Table 1. Hydrochemical characteristics of subsurface waters in research area

Borehole no	Sampling depth, m	Mineralization, mg/l	pH	Cations, mg/l				Anions, mg/l		
				K^+	Na^+	Mg^{2+}	Ca^{2+}	Cl^-	SO_4^{2-}	HCO_3^-
3	4	10602.6	7.1	56.0	3278.9	360.0	120.0	5680.0	480.0	622.2
10	3.5–4	6237.9	7.8	55.8	1485.1	324.0	60.0	2130.0	48.0	2135.0
21	9.0	9301.1	7.4	650.0	32.2	1008.0	960.0	2122.9	3613.0	915.0
25	3.5	18662.9	6.8	213.0	4359.4	1080.0	280.0	5786.5	4992.0	1952.0

Table 2. Quantity of aerobic heterotrophic bacteria in cryopegs

Borehole no.	Cultivation temperature			
	–2	5	20	37
3	n/d*	7×10^5	9.7×10^5	4.3×10^4
10	n/d	2.1×10^6	8×10^5	1.5×10^4
21	6.3×10^5	3.5×10^7	4×10^7	5.5×10^5
25	n/d	4.5×10^6	2.4×10^6	1.1×10^3

* n/d, not determined.

Methanogens were assayed by methane increase on medium 141 [8] with acetate (2 g/l) and $H_2 + CO_2$ (80:20).

The quantity of acetogens was determined in the medium containing the following (g/l): NH_4Cl, 1.0; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; NaCl, 0.9; K_2HPO_4, 0.4; yeast extract, 0.1; tryptone, 3.0; $Na_2S \cdot 9H_2O$, 0.25; cysteine, 0.4; solution of trace elements for medium 141 [8], 10 ml; $NaHCO_3$, 0.2; resazurin, 0.001; bromoethanesulfonic acid, 2.0. The substrate was $H_2 + CO_2$ (80 : 20). Acetate-forming bacteria were detected by the presence of acetate in the culture liquid.

Obtaining of enrichment and pure cultures of sulfate-reducing bacteria. The enrichment culture of strain B15 was obtained by inoculation of 20 ml of the medium with 10 ml of the cryopeg sample and incubation at 15°C. Enrichment cultures showing hydrogen sulfide production were used as inocula for further reinoculations and tenfold dilutions. The pure culture of strain B15 was obtained by the method of tenfold dilutions and inoculation in roll tubes followed by re-inoculation of the colonies into a liquid medium.

The enrichment culture of the sulfate-reducing bacterium, strain B15, was obtained in the medium containing the following (g/l): KH_2PO_4, 0.33; KCl, 0.33; NaCl, 2.0; NH_4Cl, 0.33; $MgCl_2$, 0.33; $CaCl_2$, 0.33; Na_2SO_4, 4.0; resazurin, 0.002; trace elements solution SL-10 [8], 10 ml; vitamin solution [9], 5 ml. Reducing agent (0.25 g/l of Na_2S) and lactate (2.0 g/l) were added after sterilization.

The pure culture was maintained in the medium containing the following (g/l): KH_2PO_4, 0.33; KCl, 0.33; NaCl, 2.0; NH_4Cl, 0.33; $MgCl_2$, 0.33; $CaCl_2$, 0.33; Na_2SO_4, 4.0; solution of trace elements SL-10 [8], 2 ml; vitamin solution [9], 5 ml. After sterilization, 25 ml of $NaHCO_3$ (10%) and 2.5 ml of HCl (25%) were added.

The effects of temperature, pH, and salinity on the growth of strain B15 were studied using the mineral medium containing 20 mM lactate and 20 mM sulfate. pH values from 5.3 to 8.5 were obtained using sterile solutions of 25% (vol/vol) HCl and 10% (wt/vol) $NaHCO_3$. The effect of temperature on the growth rate was studied at pH 6.9. The effect of salinity was studied in the medium containing 0, 0.1, 0.2, 0.5, 1.0, 2.0, and 3.0% NaCl. The presence of desulfoviridin was detected by the absorption peak of the cell-free extract at 630 nm. The absolute absorption spectrum was taken by a Shimadzu UV-160 double-beam spectrophotometer (Japan).

Microscopic studies. Cell morphology of the cultures was studied using a Lumam I-2 (LOMO, Russia) light microscope with phase contrast with a 90 × 15 lens and a JEM 100 electron microscope (Japan); in the latter case, negatively stained cell preparations were examined.

For negative staining, diluted cell suspensions were treated with 3% phosphotungstic acid, pH 5.0, or with 1% ammonium molybdate, pH 7.0 for 3–5 min at room temperature. Negatively stained cell preparations were examined at accelerating voltage 80 kV.

Gram staining was performed according to the conventional procedure.

Analytical methods. The acetate and methane contents were determined in a Pye-Unicam 304 gas chromatograph (Great Britain) equipped with a flame ionization detector, using the columns and modes described previously [10]. Hydrogen sulfide was detected by the colorimetric method with dimethyl-*n*-phenylene diamine [11]. The presence of bivalent iron was detected by reaction with ferrozine.

Determination of the nucleotide sequence of the 16S rRNA gene. DNA was isolated by the methods described previously [12]. Amplification of the 16 rRNA genes was performed with the universal primers 27f and 1492r in a GeneAmp PCR System 2700 (Applied Biosystems). The sequencing of the amplified 16S rDNA fragment was carried out in a CEQ2000XL automatic DNA sequencer (Beckman Coulter) using a Dye Terminator Cycle Sequencing kit (Beckman Coulter) and the protocol attached to the kit.

Phylogenetic analysis. Nucleotide sequences of the 16S rRNA genes were aligned using ClustalX software [13]. The rootless phylogenetic tree was constructed using the algorithms realized in TREECON software [14].

RESULTS

The quantity of aerobic heterotrophic bacteria determined on the 5/5 universal medium varied from tens of thousands to tens of millions of cells per ml (Table 2). Enumeration of bacteria in the samples from different boreholes at three temperatures points to the psychrotolerant nature of the community: the number of bacteria determined at 37°C is one to three orders of magnitude lower than at 5 and 20°C. In addition, we have enumerated the bacteria in a sample from borehole 21 at a temperature close to the natural temperature of the habitat (–2°C). Their quantity was 6.3×10^5 cells/ml. The colonies formed after a month of incubation were of two types. The colonies of the first type were cream-

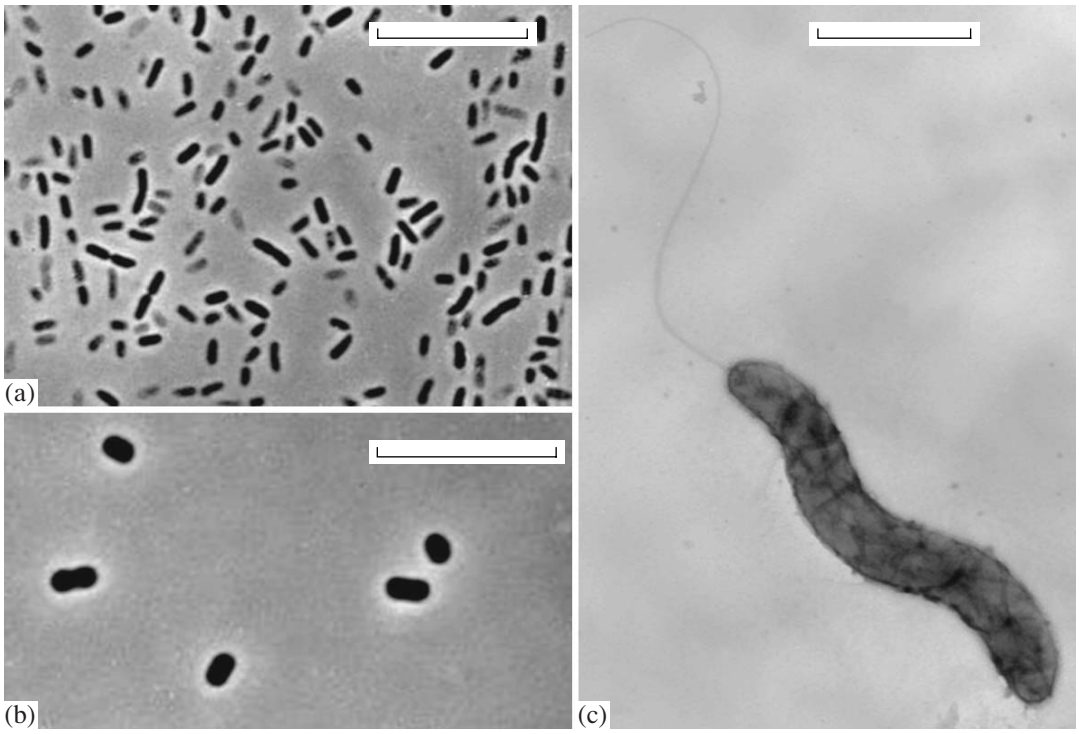

Fig. 2. Bacterial cells isolated from cryopegs of the Varandei Peninsula: (a), gram-positive rods; (b), gram-negative coccobacilli forming colonies at –2°C, phase contrast; scale bar, 10 μm; (c), negatively stained cells of the sulfate-reducing bacterium, strain B15, scale bar, 1 μm.

colored, semitransparent, with uneven edges, 5–7 mm in diameter (strain p-2). Microscopy of preparations revealed that the cells of strain p-2 were gram-negative motile rods (Fig. 2a). The colonies of the second type were white, convex, rounded, with even edges, 3–5 mm in diameter; they were formed by immotile coccobacilli, single or paired, 3.5–4 × 2–3 μm in size (strain k-2) (Fig. 2b). Morphologically, these cells resembled the cells of *Psychrobacter* sp. strains which we had isolated previously from Arctic cryopegs [2]. Partial sequencing of the 16S rRNA gene (400–500 nucleotides) from strains p-2 and k-2 showed affiliation of strain p-2 with the genus *Pseudomonas* (the cluster of fluorescent pseudomonades), whereas strain k-2 was in fact a member of the genus *Psychrobacter*.

Samples from borehole 21 were used to determine the total cell number on erythrosine-stained membrane filters, as well as the number of anaerobic bacteria of different physiological groups. The quantity of microorganisms determined by direct count was 3.5×10^8 cells/ml. As in the case with aerobes, the number of viable bacteria determined at 5°C actually did not differ from the number determined at 20°C (Table 3). The numbers of sulfate reducers and methanogens were hundreds and tens of cells per 1 ml of the sample, respectively. Acetogenic bacteria were not revealed. The numbers of halotolerant (or halophilic) members of the corresponding bacterial groups revealed on media with higher NaCl content were nearly an order lower than bacterial numbers obtained on freshwater media (Table 3).

Table 3. Total quantity and quantity of viable bacteria from different physiological groups in the sample from borehole 21

Temperature, °C	Direct count	Viable microorganisms, cells/ml				
		Aerobic heterotrophs	Anaerobic heterotrophs	Sulfate reducers	Acetogens	Methanogens
5	3.5×10^8	3.5×10^7	10^5	10^2	0	10
5*		4.3×10^7	0.5×10^4	10	0	0
20		4×10^7	10^5	10^2	0	10
20*		3×10^7	10^2	50	0	0

* The quantity was determined on the media with 40–80 g/l of NaCl.

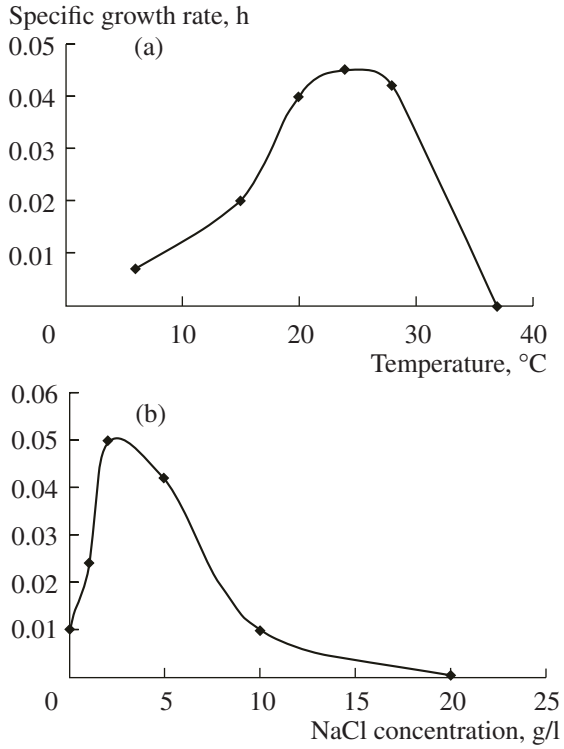

Fig. 3. Effects of temperature (a) and salinity (b) on the growth of strain B15.

The pure culture of sulfate-reducing strain B15 was obtained by the method of sequential dilutions and inoculation in roll tubes. After 2–3 weeks of incubation, strain B15 formed dark-brown colonies 2–3 mm in diameter. The cells of the strain were gram-negative vibrios, single or in short chains, 3–4 × 0.4–0.5 μm, motile due to a single polar flagellum (Fig. 2c).

The strain grew in a temperature range of 5 to 28°C (Fig. 3a). The optimal growth temperature was 24°C; no growth was observed at 37°C. Growth was observed in a salinity range of 0 to 10 g/l NaCl with the optimum at 2 g/l (Fig. 3b). The pH optimum was 6.7–7.0.

In the presence of sulfate as an electron acceptor, the strain oxidized hydrogen, formate, lactate, pyruvate, and ethanol. The strain oxidized lactate incompletely: acetate was present in the culture medium.

The strain used sulfate, sulfite, thiosulfate, and elemental sulfur as terminal electron acceptors. The strain was able to reduce ferric iron, although this process was not coupled to growth. Its growth on lactate in the presence of sulfate was stimulated by the addition of yeast extract (0.1 or 0.5 g/l) and the doubling time then decreased from 9.4 h on an unsupplemented mineral medium to 6.7 and 5.6 h, respectively.

Both desulfoviridin and cytochrome *c* (the absorption peak at 552 nm) were found in cell-free extracts.

The nucleotide sequence of the 16S rRNA gene for strain B15 contained 1425 nucleotides. The search in GenBank by BLASTn software revealed a close affinity of strain B15 to members of the genus *Desulfovibrio*. The phylogenetic tree (Fig. 4) shows the position of strain B15 within the genus *Desulfovibrio*. Strain B15 is clustered with the type strains of *D. mexicanus* and *D. aminophilus* with 96.5 and 92.0% similarity of nucleotide sequences, respectively. Strain B15 also has a rather high level of similarity in 16S rRNA with some strains of the genus *Desulfovibrio* of undefined taxonomic status, as well as with and the sequences from the clone libraries of natural microbial communities (97.2–98.1%).

Based on such phenotypic characteristics as vibrioid shape of cells, gram-negative cell wall, absence of spores, and presence of desulfoviridin and cytochrome

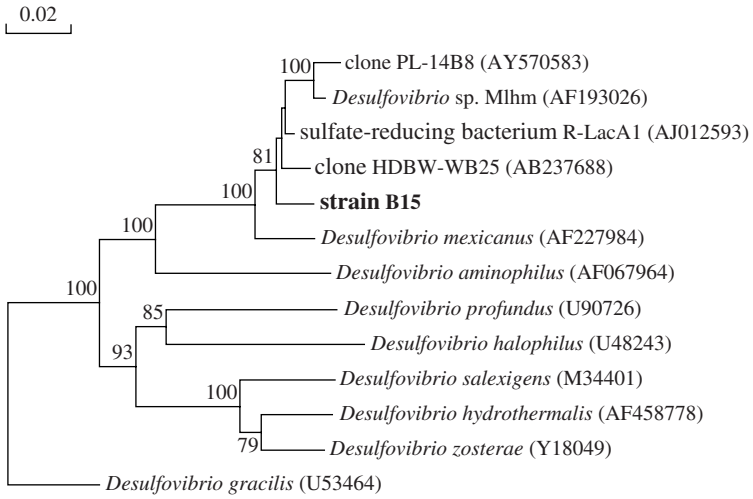

Fig. 4. Rootless phylogenetic tree of the members of the genus *Desulfovibrio* showing the position of strain B15. Numerals indicate bootstrap analysis values. The *Desulfovibrio* type strains were used for phylogenetic analysis.

c in combination with its ability to perform dissimilative sulfate reduction, as well as on the data of phylogenetic analysis, strain B15 can be assigned to the genus *Desulfovibrio*.

DISCUSSION

The results of enumeration of bacteria in the samples from different wells at different temperatures demonstrate the psychrotolerant character of the microbial community of the water-saturated horizons of the Varandei Peninsula: the quantity of aerobic bacteria detected at 37°C is the same as at –2°C (for hole 21) and one to three orders of magnitude lower than at 5 and 20°C. The number of aerobic heterotrophic bacteria determined in the water of cryopegs of the Varandei Peninsula was approximately two orders of magnitude higher than the number of anaerobic heterotrophs and did not depend on the incubation temperature (Tables 2 and 3). When incubated at 5 and 20°C, the number of halotolerant organisms was three and five orders of magnitude higher, respectively. These results differ from those we obtained for isolated highly mineralized lenses in the permafrost of the Kolyma lowland, where the numbers of aerobes and anaerobes were approximately equal [1].

Bacteria of the genus *Pseudomonas* are rather widespread geographically and are often found in low-temperature habitats, including the Antarctic [15, 16]. Quite recently, three new species of psychrophilic bacteria of this genus belonging to the group of *P. fluorescens* have been described [17]. The genus *Psychrobacter* comprises halotolerant, psychrophilic to mesophilic, immotile gram-negative coccobacilli. All members of this genus have been isolated mainly from low-temperature marine econiches [18]. *Psychrobacter cryohalolentis* 273-4 was isolated from saline cryopegs in northeastern Eurasia [19] and, due to the unusual ability of this bacterium to grow at –10°C, was selected for the determination of its complete genome. Strains 2 *Pseudomonas* sp. p-2 and 2 *Psychrobacter* sp. k-2, which were isolated under negative growth temperature, are probably psychrophiles. Further studies of the physiological and biochemical properties of these new bacteria are required to specify their taxonomic status and to determine their probable biotechnological potential.

Arctic cryopegs are characterized by a high content of sulfates. However, previous attempts to isolate pure cultures of sulfate reducers failed, probably because of the obligate dependence of these bacteria on some components of the habitat. We have succeeded in obtaining a pure culture of strain B15, which reduces sulfate when grown on lactate, formate, hydrogen, pyruvate, or ethanol, from desalinated cryopegs of the Varandei Peninsula.

There is only one publication reporting a sulfate-reducing bacterium isolated from permafrost. The gram-positive spore-forming bacterium, strain 343, was isolated from a soil sample that had been under permafrost conditions for approximately five to seven thousand years. The strain was mesophilic and showed no psychrophilic properties. The authors therefore concluded that permafrost was an environment for the preservation of this bacterium rather than for its metabolic activity [20]. Strain B15 was able to reduce sulfate at 5°C with the doubling time of 100 h. The isolated bacterium is probably able to reduce sulfates and grow in situ.

At present, the genus *Desulfovibrio* includes over 40 species. Only two of them (*D. ferrireducens* and *D. frigidus*) have been isolated from permanently cold habitats: arctic marine deposits on the western coast of the Spitsbergen archipelago. These species are also psychrotolerant mesophiles and are able to grow at the temperature of seawater freezing (–2°C) [21]. However, they are not closely related to the strain that we have isolated—the level of similarity between 16S rDNA nucleotide sequences gene does not exceed 91%.

The study of extremophilic econiches is promising with respect to the search of microorganisms with unique properties, which may be useful for biotechnology. Recently, numerous papers have been published reporting the application of psychrophilic and psychrotrophic bacteria for biotechnologies of soil purification from petroleum and other organic pollutions at high latitudes. Bacteria isolated from saline low-temperature ecosystems will probably be helpful for these purposes.

ACKNOWLEDGMENTS

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